Fish Physiol Biochem (2014) 40:983–996
DOI 10.1007/s10695-013-9899-y

Acclimation of brackish water pearl spot (*Etroplus suratensis*) to various salinities: relative changes in abundance of branchial Na^+/K^+-ATPase and $Na^+/K^+/2Cl^-$ co-transporter in relation to osmoregulatory parameters

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Received: 24 April 2013 / Accepted: 10 December 2013 / Published online: 31 January 2014

Abstract The present study was conducted to elucidate the osmoregulatory ability of the fish pearl spot (*Etroplus suratensis*) to know the scope of this species for aquaculture under various salinities. Juvenile pearl spot were divided into three groups and acclimated to freshwater (FW), brackish water (BW) or seawater (SW) for 15 days. The fish exhibited effective salinity tolerance under osmotic challenges. Although the plasma osmolality and Na^+, K^+ and Cl^- levels increased with the increasing salinities, the parameters remained within the physiological range. The muscle water contents were constant among FW-, BW- and SW-acclimated fish. Two Na+/K+-ATPase α-isoforms (NKA α) were expressed in gills during acclimation in FW, BW and SW. Abundance of one isoform was up-regulated in response to seawater acclimation, suggesting its role in ion secretion similar to NKA α1b, while expression of another isoform was simultaneously up-regulated in response to both FW and SW acclimation, suggesting the presence of isoforms switching phenomenon during acclimation to different salinities. Nevertheless, NKA enzyme activities in the gills of the SW and FW individuals were higher ($p < 0.05$) than in BW counterparts. Immunohistochemistry revealed that Na^+/K^+-ATPase immunoreactive (NKA-IR) cells were mainly distributed in the interlamellar region of the gill filaments in FW groups and in the apical portion of the filaments in BW and SW groups. The number of NKA-IR cells in the gills of the FW-acclimated fish was almost similar to that of SW individuals, which exceeded that of the BW individuals. The NKA-IR cells of BW and SW were bigger in size than their FW counterparts. Besides, the relative abundance of branchial $Na^+/K^+/2Cl^-$ co-transporter showed stronger evidence in favor of involvement of this protein in hypo-osmoregulation, requiring ion secretion by the chloride cells. To the best of our knowledge, this is the first study reporting the wide salinity tolerance of *E. suratensis* involving differential activation of ion transporters and thereby suggesting its potential as candidate for fish farming under different external salinities.

Keywords Osmoregulation · Gill · NKA α-isoforms and NKCC · Pearl spot

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Introduction

The pearl spot (*Etroplus suratensis*) is a brackish water teleost belonging to the family Cichlidae. It is widely distributed in peninsular India and Sri Lanka

and inhabits both in freshwater and in brackish water (Hora and Pillay 1962). Pearl spot is essentially a brackish water fish that has become naturally acclimated to freshwater. The species grow faster among other etroplus species and are more preferable for culture in confined brackish and freshwaters (De Silva et al. 1984; Costa 2007). Besides its high demand as food source, this species gain popularity as ornamental fishes due to their brilliant coloration. This fish shared a good percentage (8–10 %) of the total fish landings in the backwaters and in brackish water lake during the sixties (George and Sebastain 1970), which has been reduced drastically owing to overfishing (Padmakumar et al. 2002). In addition, this species is facing serious depletion in their natural habitat owing to unbridled exploitation. On the other hand, the demand for pearl spot has been increasing owing to boom of tourism in backwaters and lakes which augmented exploitation of the fishery (Padmakumar et al. 2002). Recently, pearl spot has been gaining popularity as candidate aquaculture species in inland saline regions (Kumar et al. 2009). However, seasonal fluctuations in salinity are the constraint for culture in inland saline soil. Although the fish completes its life cycle either in brackish or in freshwater, the brackish waters are the potential source of stocking materials or seeds for propagation of its culture practices in fresh and inland saline waters in spite of the fact that fry and fingerlings cannot withstand the salinity stress while transferring directly from saltwater to freshwater (Menon et al. 1959).

Euryhaline teleosts have adaptive capacity to withstand a broad range of salinities through efficient osmoregulation in order to maintain homeostasis (Evans et al. 2005). Although osmoregulation in fish is mediated by a group of organs including the intestine and kidney, the gill is the major organ responsible for balancing ion movement between gain and loss (Hirose et al. 2003). The mitochondria-rich cells (MR cells, i.e., chloride cells) located in gill epithelium are the ionocytes responsible for ion uptake in freshwater and ion secretion in seawater (Hirose et al. 2003; Hwang and Lee 2007). An extensive tubular system in cytoplasm of MR cells remains continuous with the basolateral membrane and provides a large surface area for the expression of different transmembrane proteins.

Na^+/K^+-ATPase (NKA, sodium–potassium pump) enzyme consists of three subunits: α, β and γ (Blanco and Mercer 1998). This enzyme is involved in primary ion transport by creating electrochemical gradient (Marshall 2002; Evans et al. 2005). The molecular weights of the catalytic α-subunit and smaller glycosylated β-subunit are 100 and 55 kDa, respectively (Scheiner-Bobis 2002). Besides maintaining intracellular homeostasis, NKA provides a driving force for many other ion-transporting systems (Hirose et al. 2003; Hwang and Lee 2007). Earlier studies revealed that multiple isoforms of NKA subunits are present in fish, particularly of the catalytic α-subunit (Richards et al. 2003; Bystriansky et al. 2006). Recently, differential expression of α-subunit isoforms in salmonids (Richards et al. 2003; Mackie et al. 2005; Bystriansky et al. 2006), *Chanos chanos* (Tang et al. 2009), *Anabas testudineus* (Ip et al. 2012) and *Galaxias maculates* (Urbina et al. 2013), suggests that isoform switching may be an important mechanism by which anadromous, amphidromous and other euryhaline species modulate NKA function in response to alternating salinity. Immunohistochemical studies have demonstrated that in the gills, MR cells contained most of their NKA on the basolateral membrane (Dang et al. 2000; Hwang and Lee2007). Euryhaline teleosts usually exhibit the lowest level of branchial NKA expression in salinities similar to their natural habitats (Hwang and Lee 2007). However, the gill NKA responses were influenced by their tolerance and vary with respect to different species (Kang et al. 2008; Bystrianski et al. 2006).

Among other transmembrane proteins, the $Na^+/K^+/2Cl^-$ co-transporter (NKCC) is more specifically associated with the secretory mode of gill in SW-acclimated teleost. It is a member of the cation-chloride co-transporter (CCC) family (i.e., the solute carrier family 12, SLC12) (Marshall and Bryson 1998; Hebert et al. 2004; Gamba 2005) and is crucial for maintaining plasma osmolality in euryhaline teleost in SW (Marshall 2002; Hirose et al. 2003). In euryhaline teleosts, NKCC is presumably involved in ion secretion, whereas Na^+/Cl^- co-transporter (NCC) is associated with ion absorption (Cutler and Cramb 2002, 2008; Hiroi et al. 2008). In the gills of SW-acclimated teleosts and during smolting, the abundance of NKCC protein significantly increased (Tipsmark et al. 2002; Hwang and Lee 2007) and was co-localized with NKA in gill MR cells. In teleosts, immunohistochemical staining with the T4 antibody (Developmental Studies Hybridoma Bank (DSHB), John Hopkins Univ., Baltimore, MD, USA; Lytle et al. 1995; Wu et al.

2003; Lorin-Nebel et al. 2006) on gills revealed that secretory and absorptive isoforms were localized to the basolateral membrane of MR cells in SW-acclimated fish and the apical regions of MR cells in FW-acclimated individuals, respectively (Wu et al. 2003; Hiroi and McCormick 2007; Katoh et al. 2008). To our knowledge, the osmoregulatory mechanism of pearl spot in different salinities has not been reported, which is necessary to understand their best culture environment. In the present study, we studied the status of ionic homeostasis achieved in pearl spot acclimated to different saline environments (FW, BW and SW) for 15 days. Physiological parameters (plasma osmolality; muscle water content (MWC); plasma Na^+, K^+ and Cl^- concentrations; NKA and NKCC protein expression levels; and cellular localization of NKA α-subunit and its activity) were analyzed in order to understand the osmoregulatory mechanisms of the pearl spot.

Materials and methods

Experimental animals

Pearl spot (*E. suratensis)* juveniles with an average weight of 20 ± 4.5 g were procured from Kakdwip brackish water research center, Central Institute of Brackish water Aquaculture (Kakdwip, West Bengal, India). The fishes were air lifted in a big polyethylene bag with sufficient aeration to the wet laboratory. They were carefully transferred to a circular plastic tank (1,000 L) and maintained at 10 ‰ salinity (habitat salinity) supplied with constant aeration, and one-fifth of water was replaced every 3 days. The fishes were given a dip treatment in $KMnO_4$ solution (5 ppm) next morning (Das and Mukherjee 2003). The fishes were maintained at 25 °C ± 1.5C on a light regime of 12-h/12-h light/dark cycle and 5.6–6.8 mg/l dissolved oxygen throughout the experimental period. The fishes were fed for 15 days with commercial diet containing 35 % crude protein, and to ameliorate the handling stress, the diet was enriched with vitamin C at the rate of 2,000 mg kg^{-1} (Lim et al. 2002b).

Experimental design

Juveniles ($n = 36$) with initial weight ranging from 15 to 25 g were randomly distributed in three experimental groups with triplicate following a completely randomized design. Fishes were maintained in a plastic tank of 150 L capacity (80 × 57 × 42 cm) with constant aeration, under similar salinity, light regime and water replacement patterns as described earlier. Thereafter, salinity was either increased or decreased at a rate of 2.5 ‰ day^{-1} until it reached at 1 ‰ (6.43 mOsmol kg^{-1}, denoted FW), 15 ‰ (428.45 mOsmol kg^{-1}, BW) and 30 ‰ (850.0 mOsmol kg^{-1}, SW), respectively, for each experimental group. The salinities were increased or decreased either by diluting with filtered freshwater or by exchanging with filtered seawater (35 ‰) (Seo et al. 2009). After reaching designated salinity for individual group, the fishes were fed with commercial feed twice daily for 15 days. To compare plasma parameters between the groups maintained in natural habitat salinity (BW, 10 ‰) and acclimated to BW (15 ‰), same number of juveniles ($n = 12$) were maintained in natural habitat salinity as control following similar rearing protocol described for BW-acclimated group. Thereafter, two fishes from each tank, i.e., six fishes from each salinity group, were anesthetized with clove oil (100 mg/l; Ciji et al. 2012) and sampled for blood and gill tissue samples. Rest of the fishes were kept for another experiment. During acclimatization in different salinity, the mortality was recorded every day to calculate survival rate.

Sampling

Following 15 days of acclimation in different salinity, the sampling was carried out for the analysis of the blood parameters, enzyme activity, NKA, NKCC protein identification, abundance and immunohistochemistry.

Plasma parameter

Blood was collected from the caudal vein into a heparinized syringe (no. 23). After centrifugation at 3,000 g at 4 °C for 5 min, the collected plasma was stored at −80 °C till further analysis. Plasma osmolality was estimated with cryoscopic osmometer (Osmomat 030, GonotecGmbH, Germany). The osmolality was expressed in milliosmols per kilogram (mOsmol Kg^{-1}). Plasma chloride was estimated by chloride assay kit (Abcam, USA) following their manufactures' protocol. The assay is based upon the competition between Hg^{2+} and Fe^{2+} for tripyridyltriazine (TPTZ). The preferred Hg-TPTZ adduct exhibits

no color. In the presence of chloride, Hg^{2+} forms $HgCl_2$ freeing up TPTZ which then binds the available Fe^{2+}, giving a very intense absorbance at 620 nm. The detection sensitivity of the assay was ~0.4 mM chloride. Plasma sodium and potassium were estimated by Flame Photometer (ELICO CL 378, ELICO Ltd, India).

Muscle water content (MWC)

The muscles below the dorsal fin were excised, weighed and then dried in preweighed aluminum foil for 48 h in a drying oven at 50 °C. The dried tissues were transferred to desiccators for 5 min and subsequently weighed. The muscle water content was determined from wet and dry masses and expressed as percentage of the wet weight.

Preparation of gill homogenates

Fish were anesthetized with clove oil as described earlier. The gill filaments were dissected out, blotted dry and placed in ice-cold SEI buffer (sucrose 250 mM, EDTA Na_2, imidazole 100 Mm and phenyl methyl sulfonyl fluoride (PMSF) 1 mM, pH 7.6) containing proteinase inhibitors (10 mg antipain, 5 mg leupeptin and 5 ml aprotinin; v/v 100: 1) and immediately frozen in liquid nitrogen. Gill samples were stored at −80°C for future analysis. Homogenization was performed in 2-ml tubes with a POLYTRON PT1200E (Kinematica, Lucerne, Switzerland), and 20 % (wt:vol) homogenates were then centrifuged at 13,000 g and 4 °C for 20 min. The supernatants were used for the determination of protein concentration and subsequent immunoblotting and enzyme activity. Supernatant was collected and stored at −80 °C until use. Protein concentration of tissue homogenates was estimated by Bradford (1976) method using bovine serum albumin (Fraction V, Sigma, St. Louis, MO, USA) as a standard.

Antibodies

The antibodies were NKA α-subunits (NKA, 1:500, α-5 monoclonal antibody from chicken, DSHB, USA) and $Na^+/K^+/2Cl^-$ co-transporter (NKCC, 1:500, T4 monoclonal antibody which recognizes NKCC 1 and NKCC 2 from human, DSHB, USA). β-Actin: a mouse monoclonal antibody raised against human β-actin residues and which recognized teleost counterpart (dilution 1:5,000, Abcam, Cambridge, UK, Kang et al. 2010). The secondary antibody included goat anti-mouse IgG HRP conjugate (dilution 1:2,500; Bangalore Genei, India), goat anti-mouse IgG alkaline phosphate (ALP) conjugate (dilution 1:8,000; Bangalore Genei, India) and goat anti-mouse IgG FITC conjugate (dilution 1:60; Bangalore Genei, India) both for immunoblotting of proteins and for immunohistochemical detection of NKA.

Western blot analysis

Ion transporter abundance was quantified by Western blotting as previously outlined (Pelis and McCormick2001). Samples were placed in an equal volume of 2 × lamelli buffer heated for 15 min at 60 °C and run on 7.5 % SDS–PAGE gel at 35 μg protein per lane for both NKA and NKCC and 10 μg prestained protein marker (Fermentas, USA) in a single reference lane. The proteins so separated were transferred from unstained gels to PVDF membrane (Millipore, Bedford, MA, USA) using semidry blotting apparatus (Bio-Rad, India). Blots were preincubated for 1 h in phosphate-buffered saline with Tween 20 (PBST) buffer [137 mM·NaCl, 3 mM·KCl, 10 mM·Na_2HPO_4, 2 mM·KH_2PO_4, 0.2 % (V/V) Tween 20, pH 7.4] containing 5 % (w/v) nonfat dried milk to minimize nonspecific binding. After blocking, the blot was washed with PBST, followed by 4-h incubation at room temperature with respective primary antibody of either NKA or NKCC. The same blots were incubated with β-actin antibody for normalization. The blot was washed in PBST for 5 min with three changes and was incubated either with goat anti-mouse IgG HRP conjugate or with goat anti-mouse IgG-ALP conjugate for 2 h at room temperature. The blot was further washed and then developed either with diaminobenzidine (DAB) substrate (Sigma, St. Louis, MO, USA) or with BCIP/NBT (Merck, India). The membranes so developed were photographed, and each band in individual blot was analyzed using GelPro software version 4.5 (Media Cybernetics, Inc., USA). The values for the average relative intensities of each immunodetected band of either NKA or NKCC for different salinity groups were calculated from the immunoblots of six individual samples collected from same salinity.

Immunohistochemistry

For the detection of branchial immunoreactive cells for NKA and NKCC, the first gill arch was excised, in Davidson's fixative for 24 h at 4 °C, dehydrated in ethanol and embedded in Para-plust (Sigma, St Louis, MA, USA). Serial sections (5 μm) were cut parallel to long axis of the filament and mounted on slides coated with poly-L-lysine (Sigma). Sections were dried, deparaffinized and rehydrated with ethanol and rinsed with PBS. The sections were incubated with 10 % normal goat serum in PBS for 1 h at room temperature. Slides were exposed to primary antibody (anti-α5-NKA in antibody dilution buffer) (PBS pH 7.4, 0.1 % BSA and 1 % normal goat serum) and incubated overnight at 4 °C. The slides were rinsed several times with PBS, exposed to fluorescently labeled secondary antibody at room temperature for 2 h and then rinsed several times with PBS. Slides were mounted with Flour preserve™ (Calbiochem, USA) anti-fading mounting agent. Photographs were taken in Zeiss Axio Observer A1 Fluorescence Microscope attached with Axiocam HRM camera using Axiovision software. For each fish, immunoreactive chloride cells on the primary filaments and secondary lamellae were counted from the sagittal sections of gill filaments and expressed per millimeter of primary filament. Mean number of chloride cells was obtained using the means calculated from each fish. Cell area (μm^2/cell) was obtained from immunological chloride cells using Axiovision software. At least 50 immunoreactive chloride cells from several different tissue sections were analyzed from each of six fish.

Gill Na+/K+-ATPase activity

Activity in gill homogenates was determined using a temperature-regulated microplate method (McCormick 1993). The gill homogenate supernatants were assayed for NKA activity and protein concentration. The assay solution (50 mM imidazole, 0.5 mM ATP, 2 mM phosphoenolpyruvate (PEP), 0.32 mM NADH, 3.3 U lactate dehydrogenase (LDH) mL^{-1} and 3.6 U pyruvate kinase (PK) mL^{-1}, pH 7.5) was mixed with salt solution (189 mM NaCl, 10.5 $MgCl_2$, 42 mM KCl, 50 mM imidazole, pH 7.5) in a 3:1 ratio. Samples (10 μL) in 200 μL assay buffer in the presence or absence of 1 mM ouabain were run in triplicate in 96-well microplates at 28 °C and read at a wavelength of 340 nm for 10 min on a microplate reader (BIOTEK Powerwave-340, USA). Protein concentration of tissue homogenates was estimated by Bradford (1976) method using bovine serum albumin (Sigma) as a standard.

Statistical analysis

All values are expressed as mean ± SE. Data were analyzed by one-way analysis of variance (ANOVA), and the significant differences of mean values were determined by Duncan's multiple-range test using the software program SPSS (version 11).

Results

Survival rate during acclimation to different salinities

There was no mortality, when etroplus collected from brackish water (10 ‰) and acclimated to FW, BW (15 ‰) and SW (30 ‰) for 15 days, whereas all the fish died when the salinity was increased to 35 ‰ (full-strength seawater, FSW; data not shown).

Plasma analysis and muscle water content

Plasma osmolality was lowest in FW (297.89 ± 12.0 mOsmol kg^{-1}), which increased significantly ($p < 0.05$) to 319.58 ± 11.4 and 341.52 ± 12.5 mOsmol kg^{-1}, respectively, in BW and SW groups (Fig. 1a). On the other hand, muscle water content (MWC) in BW was 80.83 ± 2.41 %, which varied in SW- and FW-acclimated individuals, but no significant difference ($p > 0.05$) in MWC among the groups was found (Fig. 1b). Plasma [Na^+] concentrations showed the similar pattern to that exhibited by plasma osmolality. The lowest [Na^+] concentration was recorded as 148.32 ± 2.61 mM in FW group, which varied with increasing salinity without marked difference among groups (Fig. 1c). The plasma [Cl^-] values were in the range of 143.23–195.21 mM and showed significant increase ($p < 0.05$) with the increasing salinity (Fig. 1c). Plasma [K^+] level was lowest (4.03 ± 0.26 mM) in FW group and that increased significantly ($p < 0.01$) to 5.19 ± 0.34 mM and 5.22 ± 0.29 mM in BW and SW groups, respectively; however, there was no marked difference ($p > 0.05$)

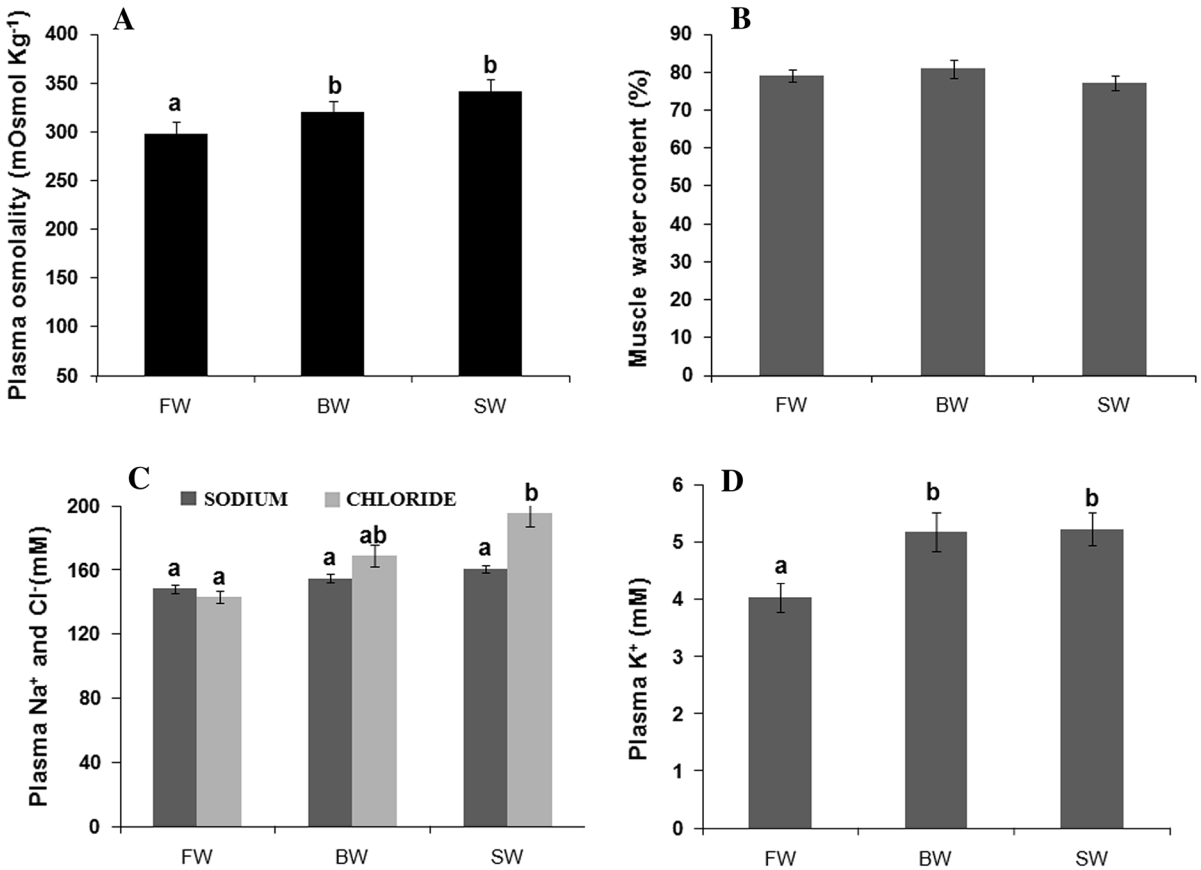

Fig. 1 Plasma osmolality (**a**), muscle water content (**b**), plasma sodium and chloride (**c**) and plasma potassium (**d**) after acclimation of *E. Suratensis* to FW, BW and SW. Data expressed as mean ± SE, $n = 6$. Mean values bearing different superscripts under each column vary significantly (ANOVA, Duncan's test, $p < 0.05$)

between BW and SW groups (Fig. 1d). Plasma osmolality and the concentrations of plasma [Na^+], [K^+] and [Cl^-] in the control group were similar to those respective values noted in BW-acclimated group (data not shown).

Gill Na+-K+-ATPase protein abundance and enzyme activity

NKA α-5 antibody detected two major bands of apparent molecular mass of 123 and 113 kDa in seawater-acclimated fish (Fig. 2a). A prominent band equivalent to 123 kDa and occasionally a second less intense band at approximately 113 kDa appeared in brackish water pearl spot (Fig. 3a). After freshwater acclimation of BW pearl spot, the abundance of NKA band of 123 kDa (band 1) decreased ($p < 0.01$), whereas it increased by 40 % after seawater acclimation (Fig. 3a, b). The second NKA band equivalent to 113 kDa was increased by 3.8- and 8.4-fold after freshwater and seawater acclimations, respectively (Fig. 3b). Gill NKA activity (3.28 ± 0.16 μ mol ADP mg^{-1} $protein^{-1}$) in BW-acclimated pearl spot was significantly lower than in FW (5.02 ± 0.28)- and SW-acclimated (7.31 ± 0.39) fish ($p < 0.01$) (Fig. 3c). The NKA protein expression in the control fish showed similar pattern as appeared in BW-acclimated fish (data not shown).

NKCC protein expression

Immunoblotting of the NKCC revealed three major bands with molecular weights of 273 ± 0.3, 222 ± 0.25 and 192 ± 0.3 kDa (Fig. 2b) in pearl spot gill extracts. The molecular weights of the immunoreactive bands appeared to be identical among

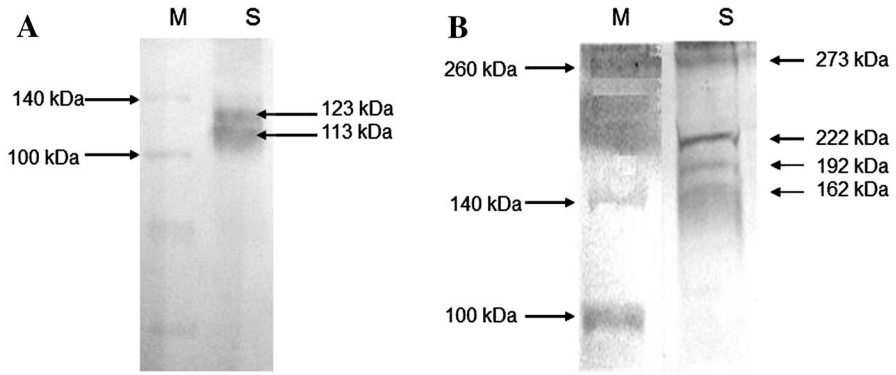

Fig. 2 Expression of NKA α-subunit protein in gills of *E. suratensis* acclimated to FW, BW or SW. **a** Immunoblots of pearl spot gills probed with the α-5 antibody indicated two bands at 123 and 113 kDa in SW groups. **b** Immunoblots of pearl spot gills probed with the (T4) NKCC antibody indicated four bands at 273, 222, 192 and 162 kDa in SW groups. *M* marker, *FW* freshwater, *BW* brackish water, *SW* seawater

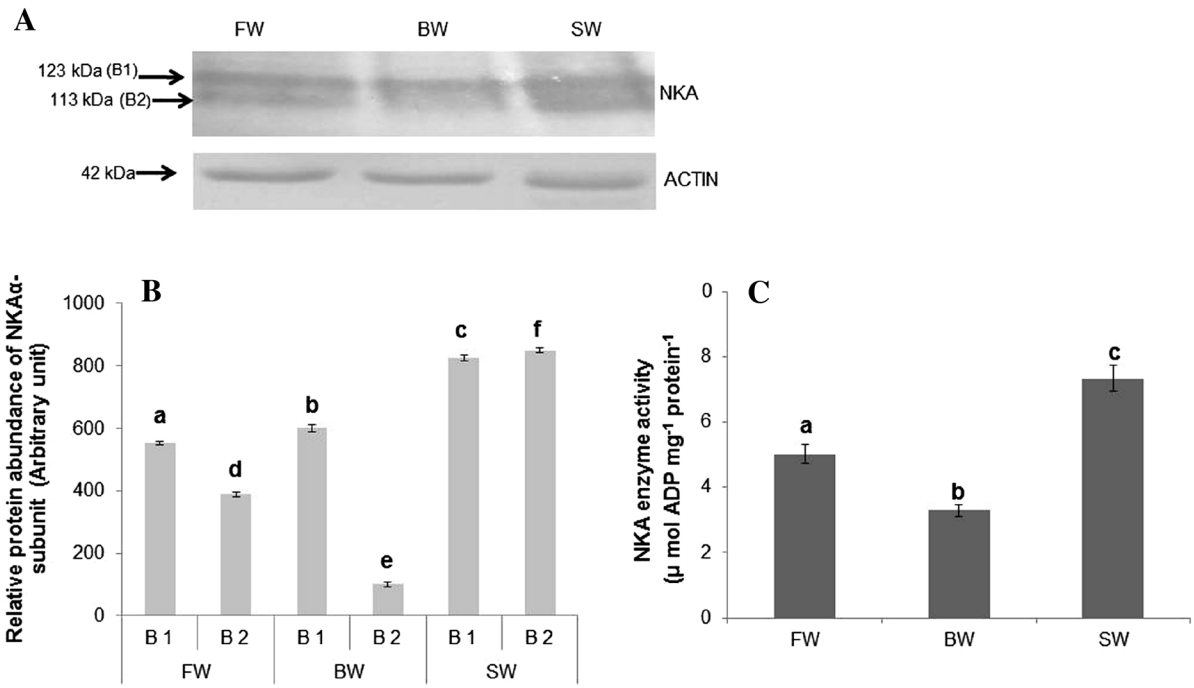

Fig. 3 Expression of NKA α-subunit protein in gills of *E. suratensis* acclimated to FW, BW or SW. **a** Immunoblots of *E. suratensis* gills probed with the α-5 antibody indicated two immunoreactive bands at 123 and 113 kDa in all environmental groups, except BW group. **b** The intensities of the immunoreactive bands of the NKA α-subunit in gills of different salinity groups ($n = 6$ for all groups) revealed that SW-acclimated *E. suratensis* was significantly higher than the other groups. **c** Gill NKA activity of *E. suratensis* acclimated to FW, BW or SW ($n = 6$ for all groups). The activity in SW-acclimated *E. suratensis* was significantly higher than in other groups. Values are mean ± SE, $n = 6$. Mean values bearing different superscripts under each column vary significantly (ANOVA, Duncan's test, $p < 0.05$). *FW* freshwater, *BW* brackish water, *SW* seawater

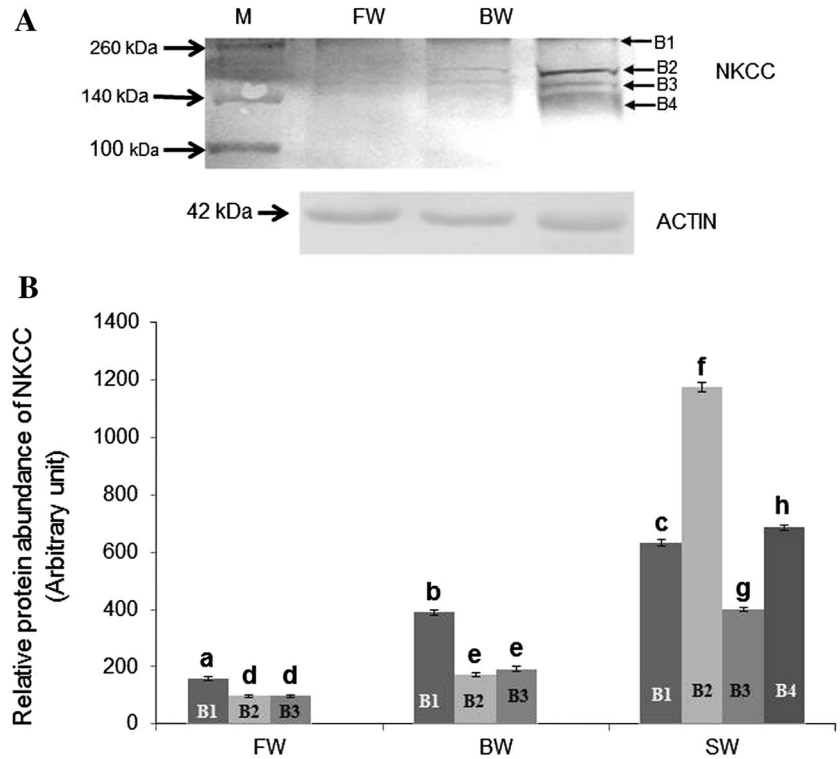

Fig. 4 Expression of Na^+, K^+, $2Cl^-$ co-transporter (NKCC) protein in gills of *E. suratensis* acclimated to FW, BW or SW. (**a**) Immunoblots of *E. suratensis* gills probed with the T4 NKCC antibody indicated three immunoreactive bands at 273, 222 and 192 kDa designated as bands 1, 2 and 3, respectively, in all environmental groups and another band, i.e., band 4, at 162 kDa in the SW groups. (**b**) The quantified intensities of the four immunoreactive bands in gills of different salinity groups ($n = 6$ for all groups) revealed that levels in SW-acclimated *E. suratensis* were significantly higher than in the other groups. Mean values bearing different superscripts under each column vary significantly (ANOVA, Duncan's test, $p < 0.05$). Values are mean ± SE. *M* marker, *FW* freshwater, *BW* brackish water, *SW* seawater

the three (FW, BW and SW groups), but the intensities of the blots varied among the groups. Image analysis of the immunoblots indicated that the 273-kDa band significantly increased approximately 2.5- and 4.0-fold higher in BW and SW groups, respectively, than those in the FW (Fig. 4a, b). Both the bands with molecular masses of 222 and 192 kDa significantly increased ($p < 0.05$) approximately 1.7- and 2-fold in BW-acclimated and 12- and 4-fold in SW-acclimated individuals, respectively, compared with their FW counterpart (Fig. 4a, b). Moreover, a lower band with molecular mass centered at 162 ± 0.25 kDa was visible only in samples from SW-acclimated fishes (Fig. 4b). The FW group showed the lowest intensity among the three groups. The NKCC protein expression in the control group was similar as appeared in BW-acclimated fish (data not shown).

Gill NKA immunoreactive cell distribution

The gill NKA immunoreactive (NKA-IR) cells in BW pearl spot were distributed in the afferent epithelium of the gill filaments (Fig. 5c), whereas NKA-IR cells were localized to cuboidal cells of various sizes on the primary filaments either at the apical region of primary filament or at the base of the secondary lamellae in FW- and SW-acclimated fish (Fig. 5a, e). The size, shape and locations indicated that these cells were MRCs. The immunoreactivity of NKA was detectable throughout cell except nucleus (Fig. 5c). The distribution pattern of MRCs was different under various salinities. MRCs were located mainly in the apical portion and scarcely in interlamellar epithelium of primary filaments in BW- and SW-acclimated fish, but

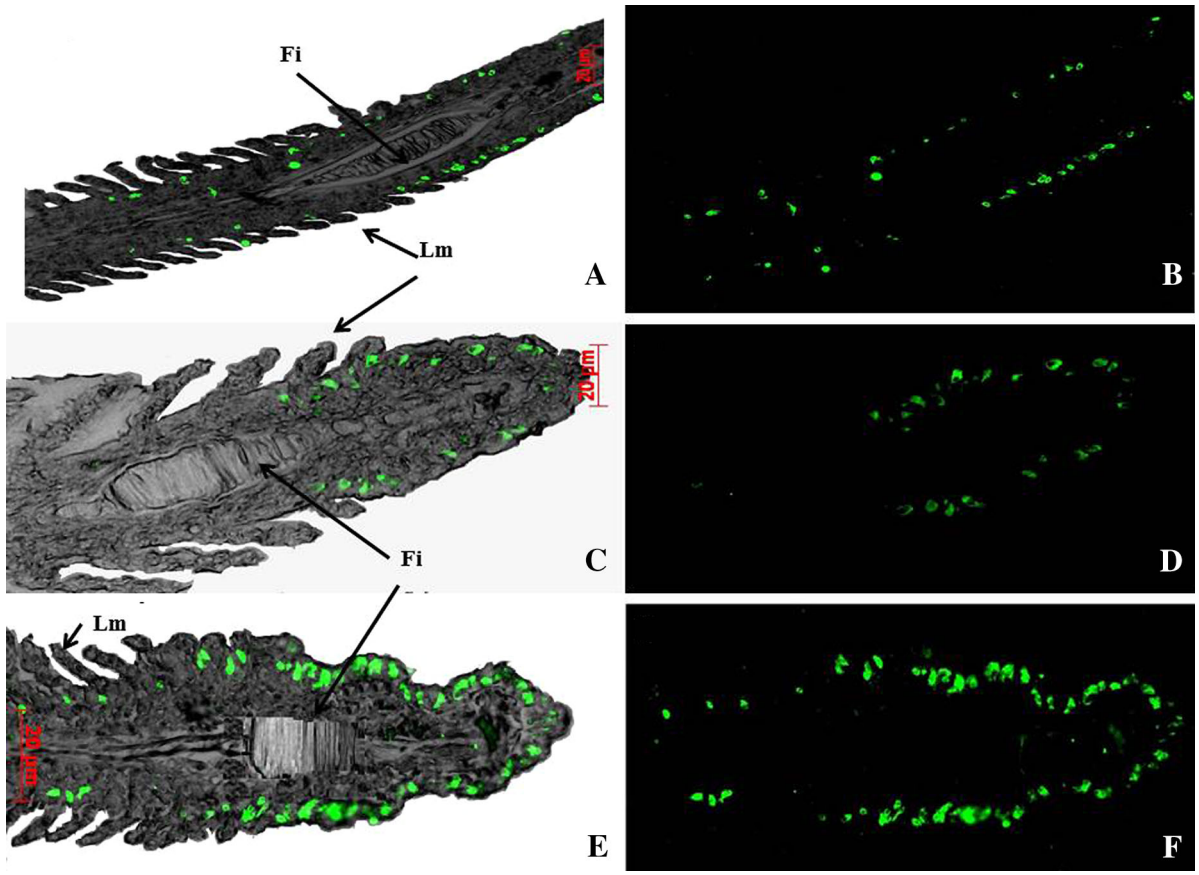

Fig. 5 Localization of Na^+/K^+-ATPase immunoreactive (NKA-IR) cells recognized by α-5 antibody in the gills of *E. suratensis* acclimated to freshwater (**a**, **b**), brackish water (**c**, **d**) and seawater(**e**, **f**). Phase-contrast micrographs (**a**, **c**, **e**) and indirect immunofluorescence micrographs (**b**, **d**, **f**) were used to demonstrate distribution of NKA-IR cells. *Fi* filament, *Lm* secondary lamellae. *Bar* = 20 µm

MRCs were distributed mainly in interlamellar epithelium of primary filaments in FW-acclimated fish (Fig. 5a, b). The size of NKA-IR MRCs in the filaments increased significantly in SW pear spot relative to BW, whereas the MRC remained smaller in size in FW-acclimated fish (Fig. 6a). The number of these cells varied significantly under salinity changes. The number was significantly lower in BW- as compared to those of FW- and SW-acclimated fish; however, the number of cells did not show marked variation between FW and SW groups ($p > 0.05$) (Fig. 6b). Neither size nor number of NKA-IR cells varied significantly ($p > 0.05$) in control group as compared to BW-acclimated fish (data not shown).

Discussion

Euryhaline teleosts display a remarkable plasticity in osmoregulatory systems in response to changes in environmental salinity. The NKA and NKCC play major roles in switching these systems efficiently to adapt in FW and SW (Kato et al. 2005). Therefore, characterization of NKA and NKCC proteins and their abundances in response to acclimation of pearl spot (*E. suratensis*) in different salinities were evaluated in this study in order to explore underlying osmoregulatory mechanisms facilitating acclimation to wide range of environmental salinities.

It is well known that pearl spot (*E. suratensis*) thrives well in freshwater and brackish water

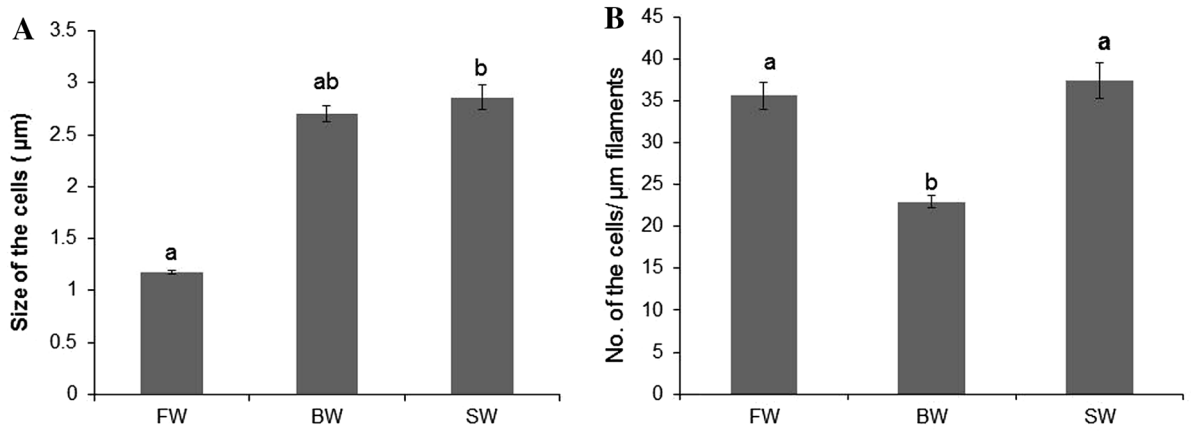

Fig. 6 Quantified average numbers of NKA-IR cells (**a**) and average sizes of NKA-IR cells (**b**) in cross sections of filaments from the gills of *E. suratensis* acclimated to FW, BW and SW. Values are mean ± SE ($n = 6$). Mean values bearing different superscripts under each column vary significantly (ANOVA, Duncan's test, $p < 0.05$)

environments (Hora and Pillay 1962). The observed survival rate in this study indicated that this brackish water euryhaline species exhibits salinity tolerance, not similar to other brackish water and marine species, such as *Chanos chanos* (Ferraris et al. 1988), *Dicentrarchus labrax* (Jensen et al. 1998), *Oryzias dancena* (Kang et al. 2008), *Galaxias maculatus* (Urbina et al. 2013) and *Monodactylus argenteus* (Kang et al. 2012), because it could not survive in full-strength seawater. In the present study, etroplus were transferred from a salinity of approximately 10 ‰ and acclimated for 15 days to FW, BW and SW (30 ‰), whereby acute ionic disturbances had become compensated. Plasma osmolality increased with increasing salinity and remained in the range of 297–341 mOsmol kg $^{-1}$, which is slightly higher than the range of 285–320 mOsmol kg $^{-1}$ normally found in euryhaline teleosts (Jensen et al. 1998; Kato et al. 2005; Kang et al. 2008). Plasma [Na+] levels and muscle water content of pearl spot did not change with salinity, whereas [K^+] and [Cl^-] concentrations became slightly higher in increasing environmental salinity; however, all those parameters stayed within physiological normal ranges as reported earlier in other brackish water teleosts (Ferraris et al. 1988; Jensen et al. 1998; Lin et al. 2003; Kato et al. 2005; Kang et al. 2008; Lundgren et al. 2008). Higher plasma parameters in SW-acclimated etroplus and mortality in FSW might occur because of deficient innate osmoregulatory ability, which was normally determined by their natural habitats (Nordlie et al. 1992), rather than stressful environment or due to gradual reduction in hypo-osmoregulatory capability of SW-acclimated fish (Yang et al. 2011). In addition, unaltered plasma parameters between BW (15 ‰)-acclimated and natural habitat (10 ‰) fishes support the influence of natural habitat on innate osmoregulatory ability of euryhaline fish. Our findings revealed that *E. suratensis* has a great capacity to acclimate under different salinities; however, that capacity may not be enough to withstand full-strength seawater.

The monoclonal anti-NKCC (T4) has been reported to recognize both the secretory (NKCC1) and absorptive (NKCC2) isoforms of NKCC from various cell types among a wide variety of species, ranging in molecular mass from 105 to 285 kDa (Suvitayavat et al. 1994; Pelis et al. 2001; Marshall et al. 2002; Wu et al. 2003; Lorin-Nebel et al. 2006). The immunoblots in the present study revealed that etroplus gills contained four major NKCC bands with molecular weights ranging from 162 to 273 kDa (Fig. 2b). Similarly, T4 immunoreacted with three major bands of molecular sizes ranging from 120 to 285 kDa in Atlantic salmon (Pelis and McCormick 2001), whereas variable T4 immunoreactive bands appeared in the range of 88–225 kDa in teleosts (Tipsmark et al. 2002; Lorin-Nebel et al. 2006; Yang et al. 2011). This variability in T4 immunoreactive bands is due to different degrees of glycosylation (Lytle et al. 1995). In FW-acclimated pearl spot, only three bands correspond to 273, 222 and 192 kDa appeared very faint, suggesting almost absence of NKCC protein

abundance in FW group. Moderate up-regulation of those three bands in the range of 1.7–12 % was observed during BW acclimation, while high abundance of those three bands and appearance of another additional band ~162 kDa during seawater acclimation provide further stronger evidence for the role of this protein in hypo-osmoregulation, requiring ion secretion by the chloride cells (Marshall and Bryson 1998; Hebert et al. 2004; Gamba 2005, Yang et al. 2011). Moreover, very low abundance of this protein in FW-acclimated gill supports that the model of ion uptake by gill epithelium in FW lacks NKCC activity (Evans et al. 1999).

Successful acclimation to hypo- and hyperosmotic media requires a net reversal of ion transport across the gill epithelium. The recent discovery of multiple Na+, K+–ATPase α-isoforms is a key step in understanding the molecular basis for this reversal (Richards et al. 2003). The relative mRNA expression patterns of two NKA α-isoforms have been investigated during salinity acclimation in a few studies [*O. mykiss*, *Salvelinus alpinus*, *Salmo salar* (Richards et al. 2003; Bystrianski et al. 2006), *O. nerka* (Shrimpton et al. 2005), *Salmo salar* (Nilsen et al. 2007; Madsen et al. 2009), *Monodactylus argenteus* (Kang et al. 2012) and *G. maculatus* (Urbina et al. 2013)]. For *E. suratensis*, the protein abundance of NKA α-band 1 (molecular mass 123 kDa) was down-regulated in the gills of fish acclimated to FW, whereas the band was up-regulated in SW, indicating that it could be a seawater isoform involving in ion absorption. By contrast, NKA band 2 (molecular mass 113 kDa) revealed an interesting pattern of abundance during salinity acclimation, and the abundance was very faint in BW-acclimated individuals and up-regulated 3.8- and 8.4-fold under FW and SW acclimations with an increase in the overall NKA enzyme activity. Both the NKA α-bands showed similar expression in control fish as appeared in BW group. Based on the reports available on differential mRNA expression of NKA *α1, α1* and *α3* isoforms in the gills of teleosts during various salinity acclimation (Feng et al. 2002; Richards et al. 2003; Urbina et al. 2013), it has been presumed that NKA *α1a* is a freshwater isoform driving ion uptake whereas NKA *α1b* is a seawater isoform driving ion secretion, and NKA *α1c* is unresponsive to salinity changes particularly in salmonids (Richards et al. 2003) whereas NKA α-1c behaves similar to the NKA α-1a in galaxiid fish (Urbina et al. 2013). In our knowledge, this is a first report of the presence of isoforms switching phenomenon in *E. suratensis* suggested by our results, which needs to be confirmed. The branchial NKA enzyme in pearl spot residing naturally in BW revealed that a U-shaped pattern activity during acclimation in FW, BW and SW environments, similar to other BW residing species, e.g., *Oryzias dancena* (Kang et al. 2008), European flounder, *Platichthys flesus* (Lundgreen et al. 2008) and sea bass (Jensen et al. 1998), supports low level of NKA expression in their primary natural habitats. The similar NKA activity in control and BW environment convincingly supports the fact. However, further detailed study is warranted to understand mRNA expression of different NKA α-isoforms and to develop specific antibodies against each of the isoforms in order to confirm the up- and down-regulation in their mRNA/protein abundance during various salinity acclimations and to determine their localization in membrane of various types of MRCs in the gills of *E. suratensis.*

Mitochondria-rich so-called chloride cells in conjunction with their neighboring cells (accessory and pavement cells) are of principal importance in ion uptake and ion excretion (Marshall 2002; Hwang and Lee 2007) and show high NKA immunoreactivity (NKA-IR) in the teleostean gill (Lee et al. 2000; Brauer et al. 2005). There is consensus that in euryhaline teleost species, ion-transporting cells may appear in both filament and lamellar positions, in some cases depending on salinity and other stressors, and also with clear species-specific differences (Uchida et al. 1996; Varsamos et al. 2002). The NKA immunoreactive (NKA-IR) cells of *E. suratensis* that acclimated to FW were distributed mainly in the epithelium of the interlamellar regions and along the afferent and efferent sides of the filament, whereas NKA-IR cells were distributed mainly near apical portion of primary filament in the BW- and SW-acclimated fish (Fig. 2) similar to distribution of MR cells in the gills of *Sartherodon melanotheron* (Ouattara et al. 2009). Appearance of ionocytes in both locations in freshwater (FW) and particularly in filament in seawater (SW) convincingly supports lamellar MRC regression and the increase in filament MRC number and particularly in size (Seidelin et al. 2000; Uchida et al. 1996). This led these authors to propose involvement of lamellar cells in ion uptake (FW–MRC) and filament cells in dual function in both ion secretion (SW–MRC) and ion uptake (Seidelin et al. 2000; Uchida et al. 1996). It is

well demonstrated that the number and size of MR cells in some euryhaline teleosts change with external salinities (Hwang and Lee 2007). The highest number and density of MR cells were reported in many teleosts such as Black Sea bream, sea bass and brackish medaka (Kelly and Woo 1999; Varsamos et al. 2002; Lin et al. 2006; Kang et al. 2008) in hypo-osmotic environment. In contrast, the present study elucidated that the number of NKA-IR cells increased in both FW and SW acclimations favors higher NKA activity and protein abundance during hyper- and hypo-osmoregulatory endurances. In general, the size of NKA-IR cells increased in SW-acclimated salmon (Hiroi and McCormick 2007); tilapia (Shiraishi et al. 1997); guppy (*Poecilla reticulate*; Shikano and Fujio 1998a, b); and *Oryza latipes* (Kang et al. 2008). The present findings deviate from earlier reports and revealed that the size of the NKA-IR cells increased not only in SW- but in BW-acclimated fish and decreased under FW acclimation.

In summary, the juvenile pearl spot inhabits in brackish water, faces wide fluctuation in habitat salinity and exhibits wide salinity tolerance between 0 and 30 ‰ through a well-developed osmoregulatory mechanism, in order to maintain plasma homeostasis within limited ranges. Expression of different isoforms of the NKA α1 subunit in *E. suratensis* is dependent on the environmental as well as habitat salinities. The expression of NKA α1b-like protein in SW and other interesting isoforms in both FW and SW is likely to facilitate switching of the osmoregulatory phenotype from ion absorbing in FW, ion excreting in SW or vice versa. The present study strongly favors that differential expression of NKA isoforms in fish is much more widespread than previously recognized (Urbina et al. 2013). Maximum NKCC protein abundance in BW and SW acclimations clearly suggests its role in hypo-osmoregulation through ion secretion, as reported earlier (Tipsmark et al. 2002). Attenuation of both the ion transporters abundance and NKA activity in FW compared with SW acclimation suggests that the pearl spot possess efficient hyper-osmoregulatory ability for adaptation. To our knowledge, this is the first report on *E. suratensis*, which clearly reveals that pearl spot is an efficient osmoregulator with differential NKA and NKCC expressions in order to maintain ion and water homeostasis in various salinity environments.

Acknowledgments The authors are thankful to Dr. W. S. Lakra, Director and Vice Chancellor, Central Institute of Fisheries Education, Mumbai, for providing necessary facilities. We acknowledge Dr. J. K. Sundaray, OIC, KRC of Central institute of Brackish Water Aquaculture, for providing the pearl spot juveniles. First author acknowledges Indian Council of Agricultural Research, New Delhi, for the Masters' fellowship.

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